Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: phenolic composition and cytotoxicity against human breast cancer cells

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Abstract

A comparison among different advanced extraction techniques such as microwave-assisted extraction (MAE), supercritical-fluid extraction (SFE) and pressurized-liquid extraction (PLE), together with traditional solid-liquid extraction, was performed to test their efficiency towards the extraction of phenolic compounds from leaves of six Tunisian olive varieties. Extractions were carried out at the best selected conditions for each technique; the obtained extracts were chemically characterized using high-performance liquid chromatography (HPLC) coupled to electrospray time-of-flight mass spectrometry (ESI-TOF-MS) and electrospray ion trap tandem mass spectrometry (ESI-IT-MS^2). As expected, higher extraction yields were obtained for PLE while phenolic profiles were mainly influenced by the solvent used as optimum in the different extraction methods. A larger number of phenolic compounds, mostly of a polar character, were found in the extracts obtained by using MAE. Best extraction yields do not correlate with highest cytotoxic activity against breast cancer cells, indicating that cytotoxicity is highly dependent on the presence of certain compounds in the extracts, although not exclusively on a single compound. Therefore, a multifactorial behavior is proposed for the anticancer activity of olive leaf compounds.

Keywords: phenolic compounds, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, breast cancer cells, HPLC-ESI-TOF-MS/IT-MS^2

1. Introduction

In the search of new bioactive compounds from natural raw materials, food by-products have gained a considerable attention for their high potential as a source of phytochemicals, low cost and high environmental impact of such residues. For instance, in the olive oil industry, one of the most promising source of bioactives are olive leaves obtained as biomass after pruning of olive trees (Erbay and Icier, 2010). This residue is a very abundant vegetable material and it supposes a potential source of polyphenols (De Leonardis et al. 2008). Constituents of olive leaves have shown antiviral (Lee-Huang et al. 2003), antimicrobial (Markin et al. 2003), antioxidant, anti-inflammatory (Bouaziz et al. 2008; Briante et al. 2002), and anti-carcinogenic (Abaza et al. 2007; Bouallagui et al. 2011) activities.

Different extraction techniques have been used to extract bioactives from olive leaves; among them, conventional solid-liquid extraction with ethanol (Rada et al. 2007) or methanol: water (Bouaziz et al. 2008) and ultrasound assisted extraction (Cárcel et al. 2010). Considering the importance of the extraction process as a way to isolate and purify interesting compounds from natural raw materials, testing different extraction procedures is mandatory. One of the main needs in the development of extraction processes is to substitute inefficient and long extraction processes, usually requiring high volumes of toxic organic solvents, for non-conventional extraction procedures such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) or pressurized-liquid extraction (PLE), that require considerably less amounts of toxic solvents while providing higher extraction efficiencies and lower environmental impact (Herrero et al. 2010, Mendiola et al. 2007). These modern extraction techniques can be regarded as a possible tool not only from a laboratory point of view but also for the natural products and food industries. In fact, industrial applications of SFE have experienced a strong development since the early 1990s in terms of patents (Schütz. 2007). It was reported that a large-scale commercial (3 tonne/hour) MAE is available for industrial use (Pangarkar. 2008). Although few reports can be found considering pilot scale units (Terigar et al. 2011; Boonkird et al. 2008), in view of the advantages of MAE and the development of equipment for large-scale commercial operation, MAE has a bright future. Thus lab scale studies can be used to determine factors required for scale-up the extraction process and equipments.

Recently, olive compounds have shown significant anti-carcinogenic effects by directly modulating the activities of various types of receptor tyrosine kinases, including the human epidermal growth factor receptor (HER2) (García-Villalba et al. 2010; Menendez et al. 2007; Menendez et al., 2009). Although secoiridoids seem to contribute importantly to such activity, the main responsible compounds have not been identified yet (Fu et al. 2010; Lozano-Sánchez et al. 2010).

Therefore, the goal of the present study was to compare different extraction processes (conventional extraction, MAE, SFE and PLE), performed under certain conditions reported in the literature, towards the selective extraction of phenolic components from olive leaves deriving from six Tunisian olive varieties. To fully characterize their phenolic composition, a new method was used based on HPLC coupled to ESI-TOF-MS and ESI-IT-MS^2. Moreover, the cytotoxicity of the different extracts against the JIMT-1 breast cancer cell line, a trastuzumab-resistant human cell line, was assayed. The possible correlation between the phenolic composition of the extracts and their cytotoxic activity was also studied.

2. Experimental

2.1. Chemicals and Reagents

HPLC-grade acetonitrile (ACN) methanol and ethanol were purchased from Labscan (Dublin, Ireland). Acetic acid was of an analytical grade (assay>99.5%) and purchased from Fluka (Switzerland). Water was purified by using a Milli-Q system (Millipore, Bedford, USA). The carbon dioxide liquefied at high pressure used in supercritical extraction was supplied by Praxair Inc. (Danbury, CT, USA).

Standard compounds such as hydroxytyrosol, luteolin, apigenin, quercetin, taxifolin, vanillin and quinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland), oleuropein and rutin from Extrasynthèse (Lyon, France).

2.2. Plant material and Treatment

Leaves used in this study were obtained from six Tunisian olive varieties: ‘Oueslati’ (1), ‘Chetoui’ (2), ‘Chemlali’ (3), ‘El Hor’ (4), ‘Jarboui’ (5) and ‘Chemchali’ (6). Olive leaves were collected from different parts of the tree, so as to minimize the sun exposure effect. After collection, fresh leaves were immediately transferred to the laboratory, washed with distilled water and ground under liquid Nitrogen.

2.3. Apparatus and instruments

2.3.1. MAE apparatus

MAE experiments were carried out with a START E Milestone Microwave Laboratory System (Milestone S.r.l, Sorisole (BG) Italy). The apparatus was equipped with a single magnetron system with rotating diffuser for homogeneous microwave distribution in the cavity, delivered microwave power was 1.200 Watts, controlled via microprocessor, allowing rapid heating of high-throughput rotors, output power up to 1200 Watts in 1 Watt increments, a Fiber-Optic Automatic Temperature Control (ATC-FO) System which allows direct continuous monitoring and control of a reference vessel up to 300°C, a MPR-600/12S medium pressure segmented rotor

containing 12 vessels for operating pressure up to 30 bar (435 psi). The microwave was operated via a compact Control Terminal 260 Interface with bright, touch-screen display.

2.3.2. SFE apparatus

The SFE system was based on a Suprex Prep Master (Suprex Corporation, Pittsburg, PA, USA) with several modifications. A thermostatic oven heated by air convection was used to set the extraction cell (8 mL) containing the sample. An HPLC pump Waters 510 (Waters Corporation, Milford, MA, USA) was used to introduce the modifier in the extraction system. A pre-heater system was employed by placing a heating coil inside a glycerine bath (JP Selecta Agimatic N, JP Selecta S.A., Abrera, Spain) to guarantee that the fluid employed in all the experiences reaches the extraction cell at the target temperature. After the modifier pump, a check valve (Swagelok SS-CHS2-BU-10, Swagelok Corporation, Solon, OH, USA) was used. A micrometering valve (Hoke SS-SS4-BU-VH, Hoke Incorporated, Spartanburg, SC, USA) was placed after the extraction cell to manually control the flowA computer-controlled mass flowmeter (EL-FLOW® Mass Flow Meter/Controller F-111C, Bronkhorst High-Tech BV, AK Ruurlo, The Netherlands) was used to adjust the carbon dioxide flow rate at the values selected for each experiment.After depressurization, the extracts were collected in a collection vessel previously described (Ibañez et al. 1999).Inside the collection vessel, 30 mL volume glass vials wereplaced to recover the extracts.

2.3.3. PLE apparatus

The PLE system consisted in a home-made device described elsewhere (Ramos et al. 2007). Basically, it consisted of an extraction cell housed in an oven provided with temperature control and regulation, a Hewlett-Packard 1050 series isocratic pump (Palo Alto, USA) to deliver and pressurize the solvent in the extraction cell and two six-port Rheodyne valves (model 7000, Rheodyne L.P., Rohnert Park, CA, USA) connected to the inlet and outlet ends of the extraction cell. The temperature and the heating rate were set by varying the energy applied to the heating resistances. The temperature programme was manually started at the beginning of each experiment and stopped at the selected extraction time. The extraction cell (8 mL) consisted in a stainless steel holder (100 mm x 4.6 mm i.d. x 6.6 mm o.d.) sealed with 5 µm stainless steel frits (Supelco, Bellefonte, USA).

2.3.4. HPLC apparatus

Separation of phenolic compounds from olive leaf extracts was performed on an Agilent 1200 series Rapid Resolution LC (Agilent Technologies, CA, USA) consisting of vacuum degasser, autosampler, and a binary pump equipped with a C18 Eclipse Plus analytical column (4.6×150 mm, 1.8 µm) from Agilent Technologies. The mobile phases used were water with acetic acid (0.5%) (mobile phase A) and acetonitrile (mobile phase B)

and the solvent gradient changed according to the following conditions: from 0 to 10 min, 95% A to 70% A; from 10 to 12 min, 70% A to 67% A; from 12 to 17 min, 67% A to 62% A; from 17 to 20 min, 62% A to 50% A; from 20 to 23 min, 50% A to 5% A; from 23 a 25 min, 5% A to 95% A; from 25 to 35 min, 95% A. The flow rate used was set at 0.80 mL/min throughout the gradient. The effluent from the HPLC column was splitted using a T-type phase separator before being introduced into the mass spectrometer. Flow entering into the ESI-TOF-MS or ESI-IT-MS detector was 0.2 mL/min. The column temperature was maintained at 25 °C and the injection volume was 10 μL.

2.3.5. ESI-TOF-MS analysis

The HPLC system was coupled to a micrOTOF (BrukerDaltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer (oaTOFMS), using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from m/z 50 to 1000. The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2 bar.

The accurate mass data of the molecular ions were processed through the newest software Data Analysis 3.4 (BrukerDaltonics, Bremen, Germany), which provided a list of possible elemental formulae by using the Generate Molecular Formula ^{TM} editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm.

During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, passing a solution of sodium formate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 (v/v). Using this method, an exact calibration curve based on numerous cluster masses, each differing by 68 Da ($NaCHO_2$) was obtained. Due to the compensation of temperature drift in the micrOTOF, this external calibration provided accurate mass values (better 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

2.3.6. IT-MS^2 analysis

An identical HPLC system was coupled to a BrukerDaltonics Esquire 2000 ion trap mass spectrometer (BrukerDaltonics, Bremen, Germany) equipped with an electrospray interface (Agilent Technologies, CA, USA) operating in the negative ionization mode. The ion trap scanned at the 50–1,000-m/z range at 13,000 u/s during the separation and detection. The maximum accumulation time for the ion trap was set at 200 ms, the target count at 20,000 and compound stability was set at 50%. The optimum values of the ESI-MS parameters were: capillary voltage, +3.0 kV; drying gas temperature, 300 °C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 21.7 psi. The instrument was controlled by Esquire NT software from BrukerDaltonics.

2.4. Extraction methods and conditions

2.4.1. Conventional solvent extraction (CM)

10 ml of a mixture of methanol and water (80:20, v/v) was added to 1 g of fresh milled olive leaves and the sample was maintained 24h in the dark at room temperature. The extracts were filtered through a 0.45µm syringe filter prior to analysis (Abaza et al. 2011).

2.4.2. Microwave-assisted extraction procedure (MAE)

1.25 g of milled fresh olive leaves were transferred into the microwave extraction vessel and suspended in 10mL of a mixture of methanol and water (80:20, v/v). Then extraction was carried out for 6 min, considering an irradiation temperature equal to 80 °C. After extraction, the vessel was cooled down to room temperature before opening, using the ventilation option of the system. The extracts were filtered through a 0.45µm syringe filter prior to analysis.

2.4.3. Supercritical fluid extraction procedure (SFE)

Prior to the extraction process, 1 g of milled olive leaves was homogenized with 1 g of sea sand, that was selected as inert material to hold the sample inside the extraction cell and to improve efficiency while avoiding formation of preferential flow paths. This mixture was introduced into the extraction cell and packed with glass wool. Extractions were carried out at 150 bar and 40°C; once reached the experimental conditions, the extraction solvent (consisting on a mixture of CO_2 plus 6.6% of ethanol as modifier) passed through the extraction cell for two hours.

2.4.4. Pressurized liquid extraction procedure (PLE)

One g of grinded olive leaves was placed in the extraction cell that was subsequently filled with 5 g of sea sand. Once the cell was mounted in the device, the selected solvent (either ethanol or water) was pumped into the cell and the lines from the pump to the outer valve. Then, the solvent was pressurized up to the selected pressure (ca.

100 bar), which was controlled via the pump recorder. Simultaneously, the temperature programme was started to heat both, sample and extraction solvent at the selected temperature (150°C) for a given time (20 min). After the static time, the upper valve was switched to allow the pressurized solvent leave the cell. Blank samples were run after each extraction to avoid any contamination or memory effect.

2.4.5. Cytotoxicity assays

The human breast carcinoma cell line JIMT-1, which derives from a breast cancer clinically resistant to trastuzumab (Tanner et al. 2004) was kindly provided by Institut Català d'Oncologia (Girona, Spain). Cells were routinely grown in DMEM + GlutaMAX medium supplemented with 10% of heat-inactivated foetal bovine serum (GIBCO) containing 50 U/ml penicillin and 50 mg/mL of streptomycin (GIBCO). Cells were incubated at 37º C in a humified 5% CO_2 air atmosphere. Cell viability was determined through the MTT assay (Fu et al. 2010). Briefly, cells were plated in 96-well plates at a density yielding 70-80 % confluence when the cytotoxicity assay was performed. Complete medium was refreshed and cultures were treated with different doses of the extracts for 72 hours. All the results corresponding to MTT experiments were expressed as the mean of a minimum of 6-8 replicates ± SD. The 50% cytotoxic concentration values (CC50) were determined from the survival plots using GraphPad PRISM 5 (GraphPad Software).

2.5. Statistical analysis

Two-way ANOVA test at a confidence level of 95% was performed using SPSS 13.0 for windows software.

3. Results and discussion

Extraction conditions were established for each extraction process considering previous results published in the literature for phenolics extraction using SFE, PLE and MAE in different matrices, including rosemary and olive leaves (Herrero et al. 2010; Herrero et al. 2011; Taamalli et al. 2011)

PLE and SFE are considered green technologies to produce bioactives from natural sources such as plants and algae (Herrero et al. 2010; Plaza et al. 2009), as GRAS-qualified solvents such as CO_2, ethanol or water are frequently used in these technologies. In this study, PLE and SFE extraction processes were carried out under optimum conditions using only GRAS-qualified solvents. These have been compared to MAE and conventional solid-liquid extraction, which used a mixture of methanol and water (80:20). Hydroalcoholic extraction is the choice of solvent for optimum phenolic's extraction due to the high extraction yield of a wide range of phenolic compounds from diverse types of samples including fruit, vegetables and olive oil (Turaet al. 2002). Our main

target was to find out advantages and drawbacks between the different extraction processes in relation to the extraction of phenolics from olive leaves.

First of all, it is worth to mention that extraction processes assisted by temperature, such as PLE and MAE, have shorter extraction times than conventional technologies and SFE; this is mainly due to the increase of the analytes solubility in the extraction media when surface tension and solvent viscosity decreases, which, at the end, improves extraction efficiency. MAE extraction temperature was selected at 80°C because, as previously reported (Taamalli et al. 2011), higher temperatures provide lower extraction efficiencies. Regarding PLE, 150°C was selected as optimum temperature for phenolics extraction when ethanol was used as extracting solvent, since this condition has been reported to achieve the highest phenolic content and antioxidant capacity (Herrero et al. 2011). Although previous results obtained in our research group suggested the use of 200°C for PLE water extractions, in this study we selected 150°C in order to avoid the generation of compounds deriving from thermal dehydration of saccharides such as 5-hydroxymethylfurfural (Plaza, M et al. 2010). On the other hand, these conditions were also optimum in terms of phenolics content, antioxidant activity and extraction yield (Herrero et al. 2011).

Table 1 shows the yield obtained for the different extraction processes using 6 different varieties of Tunisian olive leaves. A two-way analysis of variance has been carried out and the results showed that the yield was significantly influenced by the extraction method ($p<0.05$), the olive variety ($p<0.05$). The interaction of extraction method and the olive variety was also significant ($p<0.05$). As observed, the use of PLE with ethanol as extraction solvent produced the highest yield for all the studied varieties. In this sense, the highest yield was obtained from the Chemchali (6) variety. MAE also produced high extraction yields, but significantly lower than those obtained by PLE using ethanol. The only exception was the case of Jarboui (5) variety for which MAE was the less efficient extraction technique in terms of total extracted yield. Lastly, PLE using water as solvent, conventional extraction (MeOH: H_2O), as well as SFE using CO_2 and ethanol as cosolvent produced comparable yields. It is important to remark that the extraction yield strongly depends on the solvent employed. Nevertheless, extracts with similar yields, but produced under different extraction techniques, would show completely different chemical composition. Therefore, the phenolic composition of the extracts was studied in detail to evaluate the potential of the different extraction techniques.

In a recent study, we have optimized the MAE extraction conditions for phenolics' extraction from olive leaves and also identified, via HPLC-ESI-TOF-MS/IT-MS^2, the main phenolic compounds present in MAE extracts (Taamalli et al. 2011). This information and the data previously reported in literature (Fu et al. 2010; Arráez-

Román et al. 2008; Meirinhos et al. 2005; Mylonaki et al. 2008) have been used as a basis for identifying the compounds detected in the different olive leaf extracts in the present study through the comparison of their relative retention time values, TOF-MS and IT-MS^2 data in addition to the comparison with authentic standard solutions when available. Representative base peak chromatograms (BPCs) of a mass range (50–1000 *m/z*), for the extracts under the optimum extraction conditions for MAE, PLE, SFE and conventional extraction are presented (only BPCs of samples showing the highest cytotoxic activity in each extraction method are shown). Peak identification is shown in Table 2.

As can be seen in Table 2, hydroxytyrosol glucoside was not detected in 'Oueslati', 'Chetoui', 'Chemlali' and 'El Hor' varieties whereas its aglycon form (hydroxytyrosol), in addition to elenolic acid glucoside isomer 2, and luteolin rutinoside isomer 2, were not found in 'Chemchali' variety. On the other hand, secologanoside, luteolin rutinoside isomer, syringaresinol and luteolin diglucoside isomer 2 were not detected in the 'Jarboui' extract. Among the different extraction techniques used, extracts obtained under MAE conditions showed the largest number of identified phenolic compounds (Table 2). Hydroxytyrosol glucoside, secologanoside, hydroxytyrosol, elenolic acid glucoside isomer 2, vanillin and taxifolin were not detected in SFE samples from different olive leaf varieties, although non-polar compounds were extracted in a higher extent, as expected. Vanillin was not detected in the extracts obtained by PLE using ethanol as extracting solvent (PLE-E), whereas quercetin was not detected in PLE using water as solvent (PLE-W).

Quantitatively, the total phenolic contents (TPCs), expressed as the total peak areas of the identified compounds, showed variation among the different extracts according to the variety and the extraction method employed. As Figure 2 (A) shows, MAE samples showed the highest TPCs in comparison to the other extraction methods, being 'Chemlali' (MAE 3) and 'El Hor' (MAE 4) the varieties having the highest TPCs. MAE technique was followed by CM and PLE techniques, being PLE-W and SFE those achieving the lowest TPC values.

Figure 2 (B, C and D) shows the content of selected secoiridoid and flavonoid compounds, expressed as peak area, of the samples obtained with different processes and olive varieties.

Results showed that 2"-methoxyoleuropein was not well extracted with either PLE (using water or ethanol as solvent) or SFE (using CO_2 + 6.6% ethanol) as compared to the other extraction methods (Figure 2 B). SFE and PLE (using water as solvent) did not show a good efficiency either for extracting oleuropein. Besides, SFE was the best extraction procedure for apigenin and diosmetin isolation. In contrast, MAE showed the best recoveries for oleuropein, 10-hydroxy-oleuropein and 2"- methoxyoleuropein (isomer 1 and 2), being these values higher than those of 10-hydroxy-oleuropein in all the extracts, except for those extracted with PLE (Figure 2 B).

Therefore, most oleuropein derivatives seemed to be more efficiently extracted with the use of MAE or CM, being SFE the worst behaving technique. It was generally observed that considering the great chemical variability of the samples, each technique seemed to be more adequate than others for the extraction of each particular class of compounds.

Abundance of the phenolic compounds was also dependent on the variety. Among all the analyzed samples, 'El Hor' olive leaf extract obtained by SFE (SFE 4) was the richest in diosmetin (Fig. 2 C), whereas, when extracted by MAE, 'El Hor' olive leaf extract (MAE 4) was the richest in oleuropein. 'Chemlali' and 'Chemchali' extracts obtained by SFE (SFE 3 and SFE 6, respectively) were the richest in apigenin (Fig. 2 C) and luteolin (Fig. 2 D), respectively. The 'Chetoui' olive leaf extract (sample 2) was the richest in apigenin rutinoside regardless the extraction technique utilized, indicating that this variety may be especially enriched in this flavone. Besides 'Chetoui' extracts, 'El Hor' extracts obtained by using CM extraction and 'Jarboui' extracts obtained by MAE and CM were also rich in apigenin rutinoside.

Olive leaf derived compounds have demonstrated their cytotoxic activity against different cancers such as leukemia, colon and breast cancer (Abaza et al. 2007; Fu et al. 2010; Hashim et al. 2008). Their cytotoxic activity is especially relevant in HER2 positive breast cancers where olive extracts reduce the overexpression of HER2 and diminish the resistence to trastuzumab, a monoclonal anti-HER2 antibody clinically used in the therapy of these cancers (Menendez et al. 2007). JIMT-1 cells derive from a HER2 positive breast carcinoma but resistant to trastuzumab and then, constitute an adequate model to test the cytotoxic activity of olive extracts. A recent study on *Cistaceae* extracts showed a notorious capacity to inhibit the proliferation of JIMT-1 breast cancer cells (Barrajón-Catalán et al. 2010). In our study, all the extracts obtained through the different techniques were tested for their cytotoxicity on JIMT-1 cells after incubation during 72 h. Table 3 shows the CC50 values obtained. Olive leaf extracts showing CC50 values higher than 800 µg/mL were considered as non active (NA). Most of the extracts obtained by PLE (water and ethanol) or CM extraction methods showed a low cytotoxic activity with the exception of PLE-E 5. In contrast, MAE extracts showed a significant cytotoxic activity against JIMT-1 cells, especially that one deriving from 'Oueslati' variety (MAE 1). In general, the highest potency was obtained with extracts obtained by SFE. The highest cytotoxic activity corresponded to 'El Hor' olive leaf extract obtained by SFE (SFE 4), which showed a CC50 as low as 7 µg/mL. Only in a few cases, higher cytoxicity correlated to higher extraction yield (MAE 1 and PLE-E 5) (Table 1). In general, extraction yield did not correlate with the total phenolic content, revealing that other compounds different than phenolics were extracted in the processes. Whereas highest yield was obtained using PLE followed by MAE, highest phenolic

content was obtained with MAE followed by CM. Therefore, cytotoxicity seemed to be more related with phenolic compounds than with total yield. Nevertheless, most SFE extracts, which showed the lowest extraction yields i.e. < 10%, and a low total phenolic content, presented high cytotoxic activity. Thus, this activity might be due to a particular class of phenolic compounds.

As mentioned, SFE extracts were poor in oleuropein derivatives and especially abundant in flavones such as luteolin and diosmetin. Consequently, the possible influence of these compounds on cancer cell cytotoxicity deserves further attention in future studies. Anyhow, the strong cytotoxic activity observed for some extracts (SFE 4, MAE 1, and PLE-E 5) does not rely on the abundance of a single compound so the interaction of different compounds with different cellular targets is postulated to exist. In this regard, several authors have recently pointed out that dietary polyphenols may exert their pharmacological effect through their synergistic interactions by interacting with multiple targets (Efferth et al. 2011; Wagner et al. 2011). It is also plausible that the cytotoxic effect is dependent on additional compounds that were not identified in this study.

4. Conclusions

The development of new extraction processes to obtain bioactives, such as phenolic compounds, from food by-products is raising the attention of researchers and industries. A comparison has been carried out among non-conventional extraction techniques, such as MAE, SFE and PLE, and traditional solid-liquid extraction. Although the non-conventional techniques have shown important advantages, it is worth to mention that the main compositional differences among techniques depend on the type of solvent used to carry out the extraction. Thus, fast processes could be obtained using MAE and PLE, temperature-driven extraction processes, while greener processes could be achieved using SFE and PLE with water. Therefore, the optimum conditions for each process should be selected depending on the target compound to be isolated and other considerations (such as environmental impact, bioactivity, final use, etc.). In general, MAE and CM seem to be the choice for extracting more polar compounds such as oleuropein derivatives, apigenin rutinoside and luteolin glucoside isomer 3. As expected, SFE or PLE were more efficient to extract compounds with less polarity such as apigenin, luteolin, or diosmetin. The cytotoxic activity of the different olive leaf extracts against breast cancer cells does not correlate either with olive variety, process extraction yield, or amount of phenolic compounds. Highest cytotoxic effect was observed with SFE extracts, which were richer in flavones such as diosmetin or luteolin, but this biological activity does not rely on the abundance of a single compound or a family of compounds. Anyhow, the potential

anticancer activity of these compounds and their extracts, especially that of the 'El Hor' variety, deserves further attention.

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